

# Detection and Differentiation of CVI988 (Rispsens Vaccine) from Other Serotype 1 Marek's Disease Viruses

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**SUMMARY.** The serotype 1 Marek's disease virus (MDV) is the causative agent for Marek's disease (MD), a lymphoproliferative disease of chickens of great concern to the poultry industry. CVI988 (Rispsens vaccine), an attenuated serotype 1 MDV, is currently the most efficacious commercially available vaccine for preventing MD. However, it is difficult to detect and differentiate CVI988 when other serotype 1 MDVs are present. To facilitate the detection of CVI988, we developed two sets of primers for a mismatch amplification mutation assay (MAMA) PCR that targeted the single nucleotide polymorphism associated with the H19 epitope of the phosphorylated protein 38 gene. The PCR was very specific. One primer set (oncogenic primers) amplified DNA from 15 different serotype 1 MDVs except CVI988. The other primer set (CVI988 primers) amplified DNA from CVI988 but not from any of the other 15 serotype 1 MDVs. A real-time PCR assay was developed using MAMA primers, and specificity and sensitivity was evaluated *in vitro* and *in vivo*. Mixtures of plasmids (CVI988 plasmid and oncogenic plasmid) at various concentrations were used to evaluate the sensitivity/specificity of MAMA primers *in vitro*. Both primer sets were able to amplify as little as one copy of their respective plasmid. Oncogenic primers were highly specific and only amplified CVI988 plasmid when the concentration of oncogenic plasmid was very low ( $1 \times 10^1$ ) and CVI988 plasmid was very high ( $1 \times 10^6$ ). Specificity of CVI988 primers was not as high because they could amplify oncogenic plasmids when the concentration of CVI988 plasmid was  $1 \times 10^3$  and the concentration of oncogenic  $1 \times 10^2$ . Validation of MAMA primers in *in vivo* samples demonstrated that oncogenic primers can be used for both early diagnosis of MD in feather pulp (FP) samples collected at 3 wk of age and confirmation of MD diagnosis in tumors. CVI988 primers could be used to monitor CVI988 vaccination in samples with a low load of oncogenic MDV DNA (latently infected samples or negative) but not in samples with a high load of oncogenic MDV DNA (tumors). Our results suggest that monitoring CVI988 vaccination in FP samples collected at 1 wk of age ensures the specificity of the CVI988 primers.

**RESUMEN.** Detección y diferenciación de la cepa CVI988 (Rispsens Vacuna) de otras cepas del virus de la enfermedad de Marek pertenecientes al serotipo 1.

El serotipo 1 del virus de la enfermedad de Marek (MDV) es el agente causal de la enfermedad de Marek (MD), que es una enfermedad linfoproliferativa de los pollos de gran importancia para la industria avícola. La cepa CVI988 (vacuna Rispsens), una vacuna atenuada del serotipo 1 del virus de Marek, es actualmente la vacuna más eficaz disponible comercialmente para prevenir la enfermedad de Marek. Sin embargo, es difícil de detectar y diferenciar la cepa CVI988 cuando otros virus de Marek del serotipo 1 están presentes. Para facilitar la detección de la cepa CVI988, se desarrollaron dos conjuntos de iniciadores para un ensayo de PCR para la amplificación de mutaciones por discrepancia de nucleótidos (con las siglas en inglés MAMA), dirigido al polimorfismo de nucleótido simple asociado con el gene del epítipo H19 de la proteína fosforilada (PP) 38. El método de PCR fue muy específico. Un conjunto de iniciadores (primers oncogénicos) amplificó el ADN de 15 virus diferentes pertenecientes al serotipo 1 excepto la cepa CVI988. El otro conjunto de iniciadores (primers CVI988) amplificó el ADN de la cepa CVI988 pero ninguno de los otros 15 virus incluidos en el serotipo 1. Un ensayo de PCR en tiempo real fue desarrollado usando los iniciadores del ensayo MAMA y se evaluó la especificidad y sensibilidad *in vitro* e *in vivo*. Se usaron mezclas de plásmidos (plásmido CVI988 y el plásmido oncogénico) con varias concentraciones para evaluar la sensibilidad/especificidad de los iniciadores MAMA *in vitro*. Ambos conjuntos de iniciadores fueron capaces de amplificar hasta una copia de su respectivo plásmido. Los iniciadores oncogénicos fueron altamente específicos y solo amplificaron al plásmido CVI988 cuando la concentración del plásmido oncogénico fue muy baja ( $1 \times 10^1$ ) y la concentración del plásmido CVI988 era muy alta ( $1 \times 10^6$ ). La especificidad de los iniciadores para la cepa CVI988 no fue muy alta, ya que pudieron amplificar plásmidos oncogénicos cuando la concentración de plásmido CVI988 fue de  $1 \times 10^3$  y la concentración de los plásmidos oncogénico fue de  $1 \times 10^2$ . La validación de los iniciadores MAMA en muestras *in vivo* demostró que los iniciadores oncogénicos se pueden utilizar tanto para el diagnóstico precoz de la enfermedad de Marek en muestras de pulpa de la pluma (FP) recolectadas a las tres semanas de edad y para el diagnóstico confirmatorio de la enfermedad de Marek en tumores. Los iniciadores CVI988 podrían utilizarse para el seguimiento de la vacunación CVI988 en muestras con una baja carga de ADN del virus oncogénico del virus de Marek (muestras infectadas de forma latente o negativas), pero no en las muestras con una alta carga de ADN del virus oncogénico de Marek (tumores). Estos resultados sugieren que el seguimiento de la vacunación con la cepa CVI988 en muestras de pulpa de la pluma recolectadas en la primera semana de edad asegura la especificidad de los iniciadores CVI988.

**Key words:** real time PCR, poultry, avian, diagnosis, vaccine, MDV, Rispsens, CVI988

**Abbreviations:** CEF = chicken embryo fibroblast; Ct = threshold cycle; FP = feather pulp; HPLC = high-pressure liquid chromatography; HVT = turkey herpesvirus; MAMA = mismatch amplification mutation assay; MD = Marek's disease; MDV = Marek's disease virus; PI = protection index; pp = phosphorylated protein; SNP = single nucleotide polymorphism

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Table 1. MAMA primers and other primers used in this study.

Target	Primer	Sequence <sup>A</sup>
CVI988-pp38	Forward CVI988-specific	5'-GAGGGAGAGTGGCTGTCAAG-3'
Oncogenic-pp38	Forward non-CVI988-specific	5'-GAGGGAGAGTGGCTGTCA <del>AA</del> -3'
Oncogenic and CVI988 pp38	Reverse	5'-TCCGCATATGTTCCCTCCT <del>TC</del> -3'
Oncogenic and CVI988 (all serotype 1 MDV) gB	Forward	5'-CGGTGGCTTTTCTAGGTTTCG
	Reverse	5'-CCAGTGGGTTCACCCGTGA-3'
Chicken GAPDH	Forward	5'-GGAGTCAACGGATTTGGCC-3'
	Reverse	5'-TTTGCCAGAGAGGACGGC-3'

<sup>A</sup>Underlined base indicates intentional mismatch.

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by *Gallid herpesvirus 2*, though commonly referred to as MD virus (MDV). The pathogenesis of MD has been described in detail (45). MD viruses have been subdivided into three antigenically related serotypes. Serotype 3 MDV (*Meleagrid herpesvirus 1*) are the naturally occurring attenuated turkey herpesviruses (HVT). Serotype 2 MDV (*Gallid herpesvirus 3*) are naturally occurring attenuated chicken herpesviruses, and the oncogenic MDVs are classified as serotype 1 MDV (7,8).

Several vaccines are currently being used to control MD. HVT was introduced in the 1970s and continues to be used around the world today (44). As MDV evolved to greater virulence, bivalent vaccines consisting of HVT and serotype 2 MDV were required to control the new field strains (33,40). Currently, the most efficacious commercial vaccine is CVI988 (Rispen), an attenuated serotype 1 MDV (32,43).

The ability to differentiate oncogenic virus from vaccine virus is crucial for evaluating vaccine protection and to aid in disease diagnosis. This was relatively easy to do when distinguishing multiple serotypes but has become difficult since the introduction of attenuated serotype 1 vaccines. The most common approach is to PCR amplify the 132 bp repeats in serotype 1 MDV (6,34,46). Oncogenic MDV have two tandem copies of the 132-bp repeats, while cell culture-attenuated MDV typically have multiple tandem copies of the repeats (19,38). Differences, however, become more difficult to evaluate when mixed infections (CVI988 plus other serotype 1 MDVs) exist. Furthermore, originally it was believed that expansion of the 132-bp repeats was either the cause of the attenuation or was a direct consequence of attenuation. Later studies determined that the 132-bp repeat expansion was not directly involved in attenuation (35,37). It was also found that although most CVI988 viruses have multiple copies of the 132-bp repeats, some CVI988 isolates only have two copies (29,39). Consequently, a PCR based upon the 132-bp repeats might not distinguish between CVI988 and oncogenic MDV. Baigent and coworkers developed a real-time PCR for quantification of CVI988 in feather tips (3). Using their real-time PCR, they were able to monitor CVI988 virus levels in vaccinated commercial chickens (2). However, their real-time PCR amplified all serotype 1 MDV and could not be used to quantify CVI988 when other serotype 1 MDV was present.

Monoclonal antibodies have also been used to differentiate CVI988 from other serotype 1 MDV. Monoclonal antibodies T65 and H19 (28) are specific for and can be used to immunoprecipitate the viral proteins p41, p38, and p24 that make up the phosphorylated protein (pp) 38 protein complex in serotype 1 MDV (36). According to earlier published pp38 sequence data, all serotype 1 MDV strains have identical bases in the pp38 gene except bases 320 and 326 (nucleotides 1020 and 1026, GenBank accession number M74036) (15,18). H19 is specific to glutamine at amino acid 107 due to Ade at bp 320, whereas T65 is specific to glycine at

amino acid 109 due to Gua at bp 326 (16). Although all serotype 1 MDV strains except CVI988 are known to react with H19 antibody, T65 has previously only been shown to react with CVI988, GA, and three Chinese MDV field isolates. However, four MDV field isolates from Pennsylvania have also recently reacted to T65 and sequencing within the pp38 gene of the plaque-purified isolates, using an ABI 3100 Automated DNA Sequencer (Life Technologies, Carlsbad, CA), confirmed the similarity of amino acids with these previous unique strains (GA and Chinese isolates) causing the altered epitope reactivity (17).

Generally, PCRs that can differentiate between a single nucleotide difference are extremely inefficient. Kwok and coworkers determined that a single nucleotide mismatch in the 3' end of a primer resulted in moderate reduction in PCR efficiency compared with a perfectly matched primer. Single internal mismatches generally had little effect. However, multiple mismatches at the 3' end drastically reduced PCR efficiency (25). Cha and coworkers expanded upon these findings and developed a highly specific mismatch amplification mutation assay (MAMA) PCR that involves intentionally inserting a mismatched base at the penultimate position (10). In the present study, we used the MAMA PCR procedure to develop two sets of PCR primers that can be used to specifically differentiate CVI988 from other serotype 1 MDVs. The practical use of such assay includes monitoring CVI988 and diagnosis of MD under field condition when both CVI988 and oncogenic viruses are present.

The objectives of the present work was to develop a real-time PCR assay based on MAMA primers that permit differentiation of CVI988 and other serotype 1 MDV to optimize the use of this assay in biologic samples and to validate its use for monitoring CVI988 vaccination and MD diagnosis.

## MATERIALS AND METHODS

**Indirect immunofluorescence assay.** Chicken embryo fibroblast (CEF) cells were grown on cover slips and infected with MDV. When plaques appeared, the cover slips were fixed in acetone and incubated with H19 or T65 monoclonal antibody, washed with phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins. After a final wash, the cover slips were viewed with an ultraviolet microscope. The indirect immunofluorescence assay was described in detail elsewhere (28).

**PCR amplification.** All viruses were grown in DF1 cells, a continuous cell line of CEFs (23). DF1 cells in culture were infected with different strains of serotype 1 MDV, as previously described (36). At 5 to 7 days postinoculation, cells were harvested, and DNA was extracted as previously described (34).

The MAMA primers were made by MWG-Biotech Inc. (High Point, NC), purified with high-pressure liquid chromatography (HPLC), and are shown in Table 1. The forward primers were designed so the last base annealed to the first single nucleotide polymorphism (SNP) shown in Fig. 1C (bp 320). The penultimate base was changed to an A,

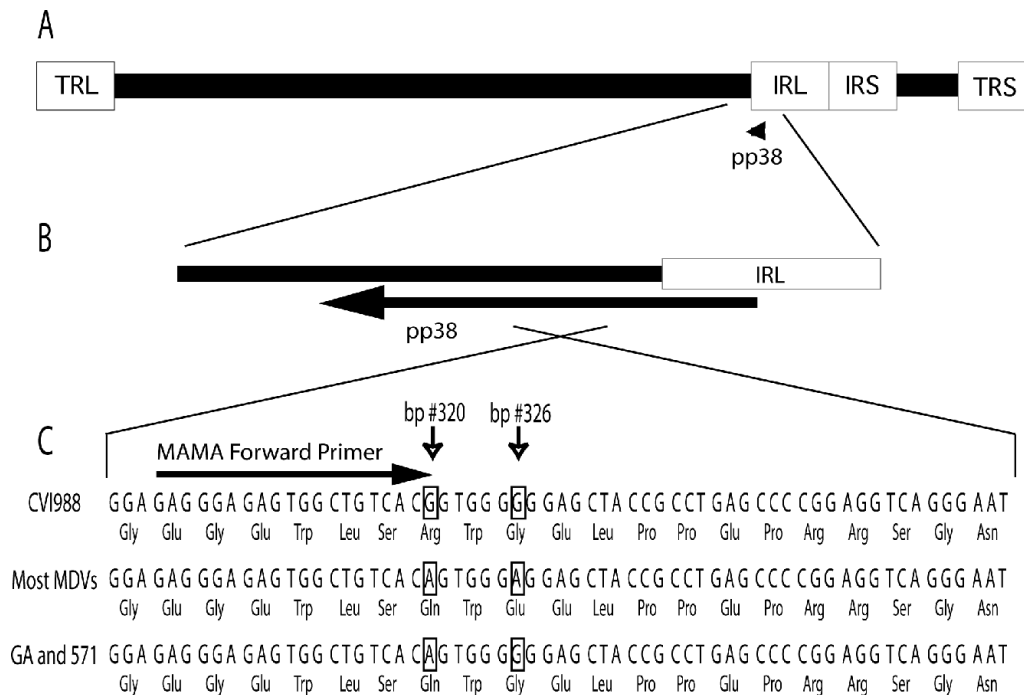


Fig. 1. SNPs differentiating CVI988 from other MDVs. (A) Schematic diagram of the MDV genome showing the terminal repeat long (TRL) and internal repeat long (IRL) flanking the unique long region (long black bar). The internal repeat short (IRS) and terminal repeat short (TRS) flank the unique short region (short black bar). The small black triangle indicates the position of the pp38 gene. (B) Expanded region of the unique long and IRL that contains the pp38 gene. The arrow indicates the position and orientation of the pp38 gene. (C) Inverted region of the pp38 gene that contains the two SNPs (boxed bases at bp 320 and 326) that differentiate CVI988 from most other MDVs. The H19 monoclonal antibody recognizes an A at position 320 and monoclonal antibody T65 recognizes a G at position 326. GA/22 and 571 are two exceptions where the second SNP at bp 326 is a G, identical to CVI988. Predicted amino acids are shown below the nucleotide sequence. Arrow indicates binding location of forward MAMA primer.

resulting in an intentional mismatch. All PCR reactions were in 50  $\mu$ l volumes and contained 20 mM Tris-HCl [pH 8.8], 2 mM  $\text{MgSO}_4$ , 10 mM KCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.1% Triton X-100, and 0.1 mg/ml nuclease-free bovine serum albumin, 2.5 units JumpStart Taq (Sigma-Aldrich Corp., St. Louis, MO), 200  $\mu$ M of each deoxynucleotide triphosphate, 25 pmol of each primer, and 25–50 ng template DNA. The CVI988-specific PCR (henceforth referred to as CVI988 PCR) consisted of the forward CVI988-specific primer and reverse primer (Table 1). Following an initial template melting step at 95 C for 3 min, the DNA was amplified during 30 cycles of 95 C for 1 min, 63 C for 30 sec, and 72 C for 20 sec. A final elongation step at 72 C for 5 min completed the PCR reaction. The non-CVI988 PCR (henceforth referred to as oncogenic PCR) used the forward non-CVI988-specific and reverse primers (Table 1). The PCR conditions were the same as for the CVI988-specific amplification except that the annealing temperature was 60 C. Seven-microliter aliquots from each PCR reaction were separated on a 2% agarose gel and stained with ethidium bromide.

**Real-time PCR.** The same primer sets described above were used for the real-time PCR assay (Table 1). In addition, primer sets specific for the gB gene of MDV (common for all serotype 1 MDV, including CVI988) and the housekeeping chicken gene GAPDH were used (21) (Table 1). Amplifications were done using the MX3005P<sup>®</sup> Stratagene (Stratagene, La Jolla, CA) in a 25  $\mu$ l PCR reaction containing 50 ng of DNA, 25 pmol of each primer, and SYBR<sup>®</sup> Green Based Master Mix (Brilliant II Fast SYBR Green qPCR Master Mix) that contains the appropriate buffers, nucleotides, and hot-start Taq-polymerase (Agilent Technology, New Castle, DE). The reaction for the MAMA primers was cycled, as indicated previously, under PCR amplification, but 40 cycles were run instead of 30. The reaction of GAPDH and gB was cycled as described (21). The melting curves for the SYBR Green amplifications were obtained at the end of amplification by cooling the sample at 20 C/sec to 60 C and then increasing the temperature to 95 C at 0.1 C/sec. The *C<sub>t</sub>* parameter (threshold cycle) was calculated for each PCR reaction

by establishing a fixed threshold. *C<sub>t</sub>* is defined as the cycle number at which the fluorescence passes the fixed threshold. Relative quantification of the load of MDV DNA was evaluated by the comparative *C<sub>t</sub>* method as previously reported (21). Total quantification (number of DNA copies per 50 ng of total DNA) was also achieved by using standard curves. Serial dilutions of plasmid samples (see next section) were used to develop standards curves for CVI988 and for oncogenic MDV.

**Validation of real-time PCR assay using plasmid samples.** The MAMA primers were validated for accuracy and sensitivity using plasmid constructs. Two plasmids were generated using the Zero Blunt PCR cloning kit (Life Technologies), each containing the unique pp38 gene with a different bp 320. The first plasmid was generated using the A6 cosmid, one of five overlapping cosmid clones used to create the recombinant clone rMd5 (30). The second plasmid was generated using DNA isolated from rMd5/pp38CVI, a recombinant MDV strain with the pp38 gene from CVI988 inserted into rMd5 (27). First, plasmid stocks were serially diluted in salmon sperm DNA (2.5 ng/ $\mu$ l) from  $1 \times 10^6$  copies/ $\mu$ l to 1 copy/ $\mu$ l for both plasmid stocks. Each serial dilution was independently evaluated using both MAMA primers for sensitivity and specificity. Second, the two plasmids were combined together in mixtures using the serially diluted stock in a matrix that included each of the seven dilutions for one stock combined with each of the seven dilutions from the second plasmid stock. Each of these mixtures was evaluated independently as above using both MAMA primers. All real-time PCR reactions were run in triplicates.

**Validation of real-time PCR assay using biologic samples.** Validation of MAMA primers in biologic samples was done in two steps (Table 2). First, we evaluated the use of MAMA primers in chickens that have been infected either with CVI988 (and no challenge) or with oncogenic MDV (but no CVI988). This step allowed us to determine if MAMA primers were sensitive/specific enough to detect CVI988 DNA in the feather pulp (FP) under different conditions and oncogenic MDV DNA in latently infected tissues and tumors. In addition, we optimized the

Table 2. Validation of MAMA primers in biologic samples.

Step	Infection	Objective	Sample <sup>A</sup>	Origin of samples <sup>B</sup>	No. <sup>C</sup>
1	CVI988 only	To optimize the use of CVI988 primers in unchallenged chickens	FP	Experiment 1	44
			FP	Experiment 2	64
	Oncogenic and serotype 2 and 3 vaccines (no CVI988)	To optimize the use of oncogenic primers in chickens nonvaccinated with CVI988	LIT	Various experiments (21,22)	12
			MDT	Various experiments (21,22)	12
	Oncogenic and serotype 2 and 3 vaccines (no CVI988)	To optimize the time of sampling for the use of MAMA primers	FP	Experiment 3	150
2	CVI988 and oncogenic MDV	To validate MAMA primers to monitor CVI988 vaccination in FP of 7-day-old chickens	FP	Experiment 4	150
			FP	Experiment 5	150
			FP	Experiment 6	100
			FP	Experiment 7	100
			FP	Experiment 8	100
		To validate MAMA primers for the early diagnosis of MD (3 wk)	FP	Field samples	190
			FP	Experiment 6	120
			FP	Experiment 7	120
		To validate MAMA primers for diagnosis of MD	FP	Experiment 8	120
			Tumors	Field cases	99

<sup>A</sup>Type of sample used for the assay. FP = feather pulp; LIT = latently infected tissues; MDT = MD virus-induced tumors.

<sup>B</sup>Samples from various experiments as well as from field cases were used. When available, references are provided. Details of Experiments 1–8 are provided in Table 3.

<sup>C</sup>Number of samples used for the present study.

time for sampling when MAMA primers are used. Those optimum times for sampling were used in the second step, which was to validate the use of MAMA primers in chickens that were infected with both CVI988 and oncogenic MDV and to determine the practical application of this assay.

A retrospective study using samples obtained in various experiments, as well as field case samples, submitted for diagnosis/monitoring vaccination to our laboratory was done. Information about the samples used, as well as the origin of the samples, is presented in Tables 2 and 3. Table 2 indicates the number and type of sample used, the rationale why those samples were used, and the experiment from which the sample was obtained. Table 3 summarizes the details of the experiments from which samples were obtained.

*Step 1: Validation of real-time PCR assay in chickens vaccinated with CVI988 and unchallenged or in chickens challenged but not vaccinated with CVI988.* A retrospective study using samples collected in various experiments was conducted to validate the MAMA primers in chickens that have been infected either with CVI988 (and no challenge) or with oncogenic MDV (but no CVI988).

To optimize the use of CVI988 primers, we used 108 samples from two different experiments (Experiments 1 and 2) in which meat-type chickens were inoculated with various doses of CVI988. In Experiment 1, CVI988 was administered *in ovo* at 18 days of embryonation and in Experiment 2 at 1 day of age via subcutaneous route. Samples of FP in

both experiments were collected at 1, 3, and 8 wk of age. Details about the samples used and the experimental design are in Tables 2 and 3.

To optimize the use of oncogenic primers, we used 12 tissue samples latently infected with oncogenic MDV and 12 samples of tumors induced by oncogenic MDV. None of the samples used came from chickens vaccinated with CVI988. Samples were selected from various experiments described somewhere else (21,22) and are samples that we used in our laboratory as a control for MDV-induced latency and MDV-induced tumors.

To optimize the time of sampling for using MAMA primers to monitor CVI988 vaccination and for diagnosis of MD, we used 450 FP samples from three different experiments (Experiments 3–5) in which chickens were unvaccinated or vaccinated with vaccines of serotypes 2 and 3 and challenged with either 648A or Md5. Details about the samples used and the experimental design are in Tables 2 and 3.

*Step 2: Validation of MAMA primers to monitor CVI988 replication and the diagnosis of MD in chickens exposed to both CVI988 and oncogenic MDV.* We used the validation of MAMA primers to monitor CVI988 replication in FP. A retrospective study was conducted using 300 FP samples from Experiments 6–8 in which CVI988 was administered at the manufacturer's recommended dose. In the three experiments, chickens were 7 days old at the time of the sampling. In addition, 190 FP samples from five commercial flocks submitted to our laboratory for monitoring CVI988 replication were used. All field samples used in this

Table 3. Experiments from which samples were retrospectively collected for this study.

Expt. <sup>A</sup>	Chicken <sup>B</sup>	MD vaccine <sup>C</sup>			MDV challenge <sup>D</sup>		Sampling <sup>E</sup>	
		Strain	Dose (plaque-forming unit)	Age/route	Strain	Age/route	Tissue	Age
1	MT	CVI988	2000–4000	18ED/amn	NA	NA	FP	1, 3, 8 wk
2	MT	CVI988	800–6400	1 day/SC	NA	NA	FP	1, 3, 8 wk
3 and 4	MT	HVT HVT+SB-1	2000–4000	1 day/SC	648A	1d/contact	FP	1, 3, 8 wk
5	MT	HVT HVT+SB-1	2000–4000	1 day/SC	Md5	1d/contact	FP	1, 3, 8 wk
6 and 7	MT	CVI988	2000–4000	18ED/amn	648A	1d/contact	FP	1, 3 wk
8	ET	CVI988	2000–6500	1d/SC	648A	1d/contact	FP	1, 3 wk

<sup>A</sup>Samples from Experiments 1–8 were used in this study. Details of the experiments are presented in this table. See Table 2 for the number of samples used and for what the samples were used.

<sup>B</sup>MT = meat-type chicken; ET = egg-type chicken.

<sup>C</sup>18ED = 18 days of embryonation; amn = amniotic route; SC = subcutaneous route.

<sup>D</sup>NA = not administered.

<sup>E</sup>FP = feather pulp.



study came from meat-type chickens vaccinated with CVI988 *in ovo* or at 1 day of age and were also collected at 7 days of age.

We used the validation of MAMA primers for the early diagnosis of MD. A retrospective study was conducted using 360 FP samples from Experiments 6–8. All samples were collected at 3 wk of age and came from chickens vaccinated *in ovo* or at 1 day of age with CVI988 and challenged with 648A at 1 day of age by contact. We selected samples from treatment groups that had different level of protection at the termination of the study (8 wk) based on the presence of tumors in viscera and nerves. Load of oncogenic MDV DNA was evaluated using oncogenic primers to show that they could be used for an early diagnosis of MD, as reported previously (21). The percentage of chickens with tumor levels (ratio *Ct* GAPDH/*Ct* oncogenic  $\geq 1.17$  based on results obtained in the previous section) was evaluated in the different treatment groups. The percentage of tumors in the FP samples collected at 3 wk was correlated with the protection index (PI) obtained at the end of the experiments (8 wk) for that particular treatment group. The PI is calculated based on the presence of gross tumors in nerve of viscera during the necropsy. The formula is as follows:

$$PI = (\% \text{ tumors in the positive control} - \% \text{ tumors in the Txt}) / (\% \text{ tumors in the positive control} \times 100).$$

Ninety-nine tumor samples from clinical cases submitted to our laboratory were used for the diagnosis of MD in tumor samples. MAMA primers were used in comparison with the gB primers previously described (11,12,21,22) and histopathology.

**Analysis of the data and statistical analysis.** Data were analyzed using the statistical program Statistica® (Statsoft, Tulsa, OK). The Pearson correlation test was conducted to evaluate lineal correlation between two variables. Comparison between groups was conducted by Student *t*-test. The level of statistical significance considered was at  $P < 0.05$ .

## RESULTS

**Specificity of monoclonal antibodies H19 and T65.** To confirm the reported lack of specificity of H19 and T65, we performed an indirect immunofluorescence assay on CEF cells infected with a variety of serotype 1 MDVs. The results of the immunofluorescence assay are shown in Table 4. As expected, H19 reacted with all the tested MDV except for CVI988. T65 was positive with CVI988, GA/22, 571, and six recent field isolates (701, 703, 709A, 710, 718, and 723) obtained from Pennsylvania farms and was negative with the other MDV. Our findings confirm earlier reports that T65 reacts with GA/22 (13). DNA sequencing of CVI988, GA/22, 571, and two of the field isolates indicated that they all contained a G at pp38 bp 326.

**DNA amplification.** The forward MAMA primers (Table 1) bind to the pp38 gene with the last base annealing to the SNP at bp 320 (Fig. 1C). An intentional mismatch was inserted in the penultimate base of the forward primers (C to A). PCR amplification with the CVI988 and oncogenic primers are shown in Fig. 2. The CVI988 primers failed to amplify 16 different serotype 1 MDVs (Fig. 2A) but did amplify DNA from samples containing CVI988 (Lane 19) and two DNA preparations that contained both CVI988 and either Md5 or 648A (Lanes 20 and 21). The oncogenic primers amplified all 16 different MDVs (Fig. 2B) but not CVI988 containing DNA (Lane 19). The oncogenic primers also amplified DNA from preparations containing a mixture of CVI988 and either Md5 or 648A (Lanes 20 and 21).

**PCR sensitivity.** To determine the sensitivity of the PCRs, we made serial dilutions of pp38-containing plasmids. One pp38 plasmid contained the CVI988 SNPs (G-G; CVI988 plasmid), while the other plasmid had the non-CVI988 SNPs (A-A; oncogenic plasmid). The oncogenic primers amplified as little as one copy of

Table 4. H19 and T65 immunofluorescence staining of serotype 1 MDVs.

MDV strain	Pathotype <sup>A</sup>	H19 <sup>B</sup>	T65
JM/102W	v	+	–
GA/22	v	+	+
270/1	v	+	–
272A	v	+	–
279C/1	v	+	–
279M/1	v	+	–
286	v	+	–
293-12/1	v	+	–
295	v	+	–
571	v	+	+
RB1B	vv	+	–
Md5	vv	+	–
Md11	vv	+	–
584A	vv+	+	–
648A	vv+	+	–
CVI988	attenuated	–	+
701	vv	+	+
703	vv	+	+
709A	vv+	+	+
710	ND <sup>C</sup>	+	+
718	vv	+	+
723	vv	+	+

<sup>A</sup>Pathotype determination described by Witter (43). m = mild; v = virulent; vv = very virulent; vv+ = very virulent plus.

<sup>B</sup>Results of indirect fluorescence test with monoclonal antibodies H19 and T65; + = positive. – = negative.

<sup>C</sup>Pathotype ND = not determined.

the oncogenic plasmid, but they also amplified CVI988 plasmid when the concentration was  $1 \times 10^4$  copies or more (Fig. 3). CVI988 primers were able to detect as little as one copy of the CVI988 plasmid, but they also amplified the oncogenic plasmid when the concentration was  $1 \times 10^2$  copies or more (Fig. 3).

Sensitivity and specificity of the MAMA primers were also evaluated in mixtures of the two plasmids in which a fixed amount of one of the plasmids was mixed with serial dilutions of the other plasmid ( $1 \times 10^6$  to  $1 \times 10^0$ ; Fig. 4). CVI988 primers were able to detect the proper amount of CVI988 plasmid present in the mixture when the load of CVI988 plasmid was  $1 \times 10^4$  or higher. When the load of CVI988 plasmid was of  $1 \times 10^3$ , CVI988 primers could still detect the proper amount of CVI988 present in the mixture as long as the load of oncogenic plasmid was not higher than  $1 \times 10^2$  (Fig. 4A). Oncogenic primers were able to detect the proper amount of oncogenic plasmid present in the mixture as long as the load of oncogenic plasmid was  $1 \times 10^2$  or higher. When the load of oncogenic plasmid was of  $1 \times 10^1$ , oncogenic primers could still detect the proper amount of oncogenic present in the mixture as long as the load of CVI988 plasmid was not higher than  $1 \times 10^6$  (Fig. 4B).

**Validation of real-time PCR in biologic samples.** *Optimization of CVI988 primers in samples of chickens challenged but not vaccinated with CVI988.* To determine if CVI988 DNA load reaches the level that could interfere with the sensitivity/specificity of the oncogenic primers (load of CVI988  $\geq 1 \times 10^6$ ) with *in vivo* samples, we used CVI988 primers to evaluate the load of CVI988 in 108 FP samples obtained in two different experiments (Experiments 1 and 2; see Tables 2, 3). Samples tested came from chickens that had been vaccinated with CVI988 either *in ovo* or at 1 day of age with various doses of the vaccine. In addition, samples had been collected at different ages (1, 3, and 8 wk of age). Load of CVI988 DNA in the samples tested ranged from 1 to 44 copies per 50 ng of total DNA; however, the majority of the samples were below 5 copies per 50 ng

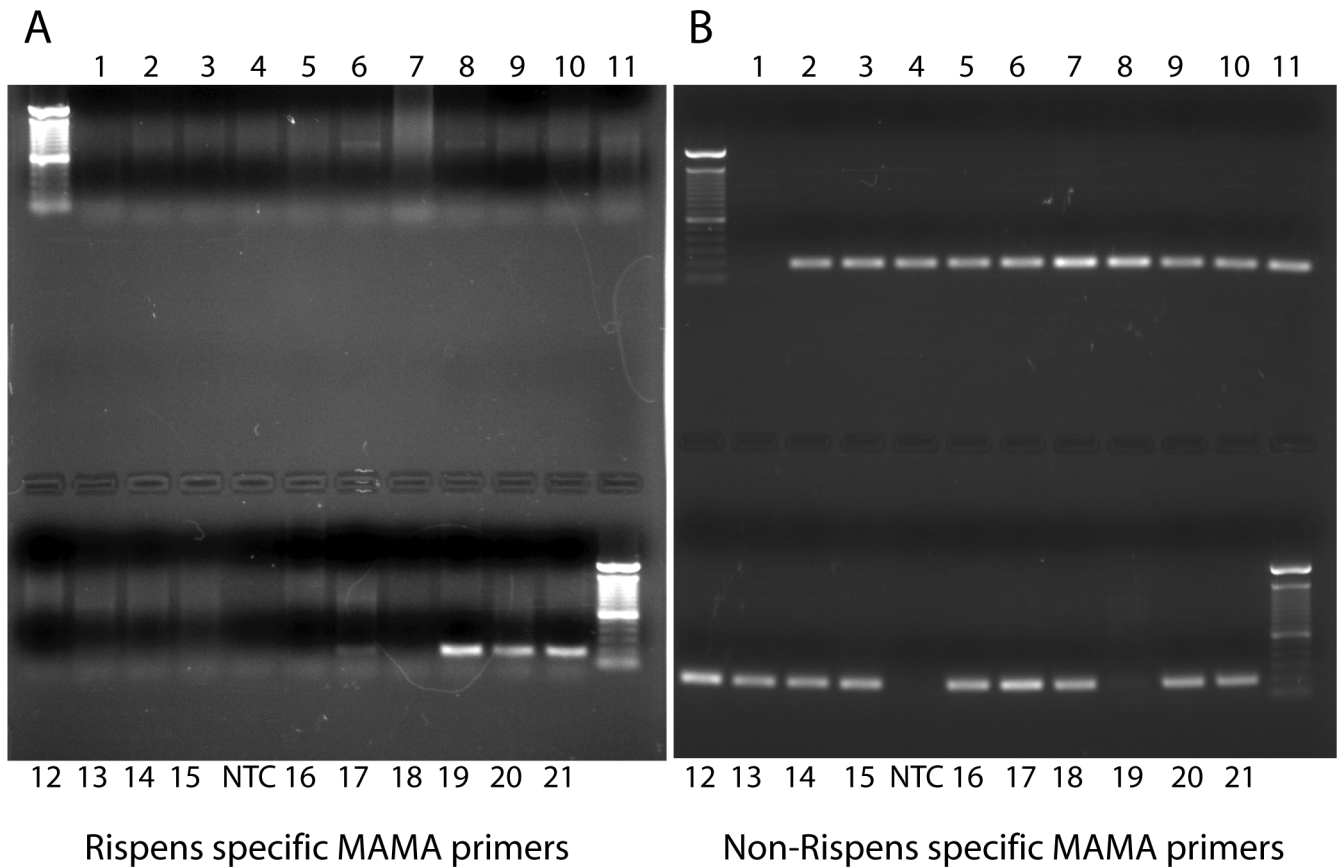


Fig. 2. Ethidium bromide-stained agarose gel of MAMA PCR products. Lane 1: DNA from DF1 cell line (no MDV); Lane 2: CU2; Lane 3: 571; Lane 4: 596A; Lane 5: 617A; Lane 6: JM/102W, cell culture passage 22; Lane 7: RB1B; Lane 8: GA/22; Lane 9: 595; Lane 10: 549A; Lane 11: Md5; Lane 12: Md11, cell culture passage 16; Lane 13: 645; Lane 14: 648A; NTC, no template control; Lane 16: Md11/75C; Lane 17: Md11, cell culture passage 102; Lane 18: JM/102W, cell culture passage 222; Lane 19: CVI988; Lane 20: CVI988 and Md5; Lane 21: CVI988 and 648A. Panel A: PCR products using Rispsens- specific (CVI988) MAMA primers. Panel B: PCR products using non-Rispsens-specific (CVI988) MAMA primers. First and last lanes in each panel contain 100-bp DNA molecular weight markers from Invitrogen Corp., Carlsbad CA.

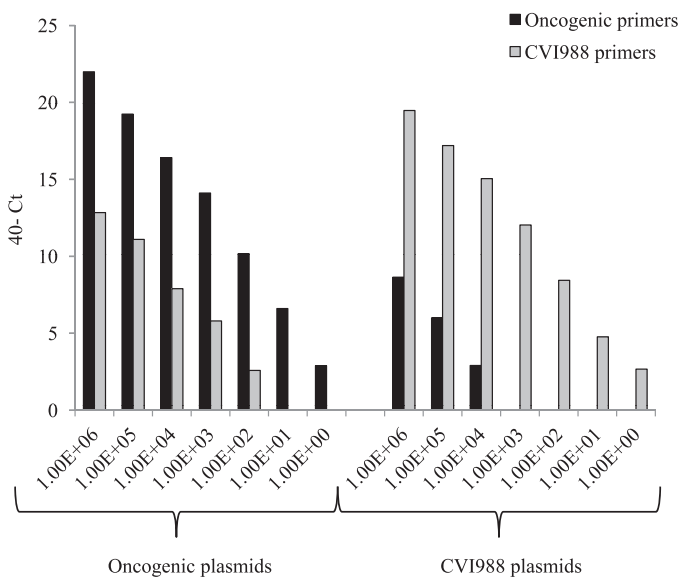


Fig. 3. Validation of MAMA primers in oncogenic and CVI988 plasmids. Oncogenic (black bars) and CVI988 (grey bars) primers were used to amplify serial dilutions ( $1.00 \times 10^6$  to  $1.00 \times 10^0$ ) of either oncogenic or CVI988 plasmids. Results are presented as total number of cycles minus threshold line  $Ct$  ( $40 - Ct$ ).

of total DNA (Fig. 5). To confirm that oncogenic primers could not detect such low levels of CVI988, we used the oncogenic primers in the same 108 FP samples. No amplification was detected (data not shown). Therefore, *in vivo* levels of CVI988 are not high enough to interfere with the sensitivity/specificity of the oncogenic primers.

*Optimization of oncogenic primers in samples of chickens challenged but not vaccinated with CVI988.* To determine if oncogenic MDV DNA load reaches the level that could interfere with the sensitivity/specificity of the CVI988 primers (oncogenic levels  $\geq 1 \times 10^2$ ) with *in vivo* samples, we used 24 samples from chickens that were not vaccinated with CVI988. Twelve of those samples were latently infected tissues and the other 12 were tumors. The criterion used to classify those samples into latency or tumors was real-time PCR based on gB amplification previously described (11,12,21,22). Briefly, samples latently infected had a ratio  $CtGAPDH/CtgB$  lower than 1.7. Tumor samples had a ratio  $CtGAPDH/gB$  higher or equal to 1.7. Oncogenic primers detected up to 9.01 copies of MDV DNA in the latently infected tissues and between 156 and 858 copies of MDV DNA in the tumor tissues (Table 5). The ratio  $CtGAPDH/Ct$  oncogenic was 0.78–0.99 for latently infected samples and 1.17–1.45 for tumor samples. No amplification was observed in the latently infected samples when CVI988 primers were used. However, up to four copies were detected in the tumor samples with the CVI988 primers, even though CVI988 was not present in those samples. These results indicate that load of oncogenic MDV

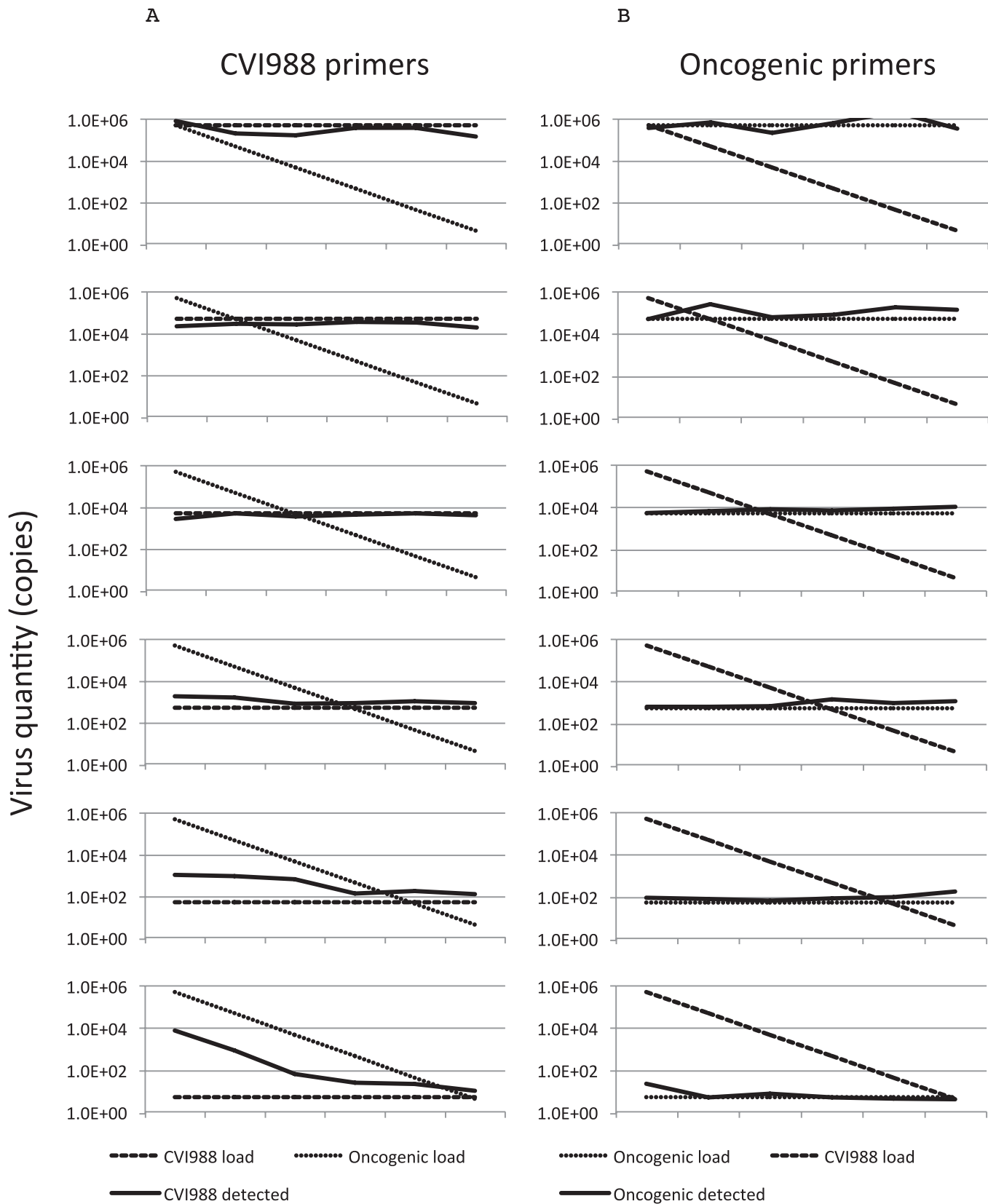


Fig. 4. Validation of MAMA primers in mixtures of oncogenic and CVI988 primers. (A) A fixed amount of CVI988 plasmid was mixed with serial dilutions of the oncogenic plasmid, and mixtures were amplified with CVI988 primers. The expected level of CVI988 present in the mixture (dashed line) was compared with the detected level of CVI988 (black solid line). CVI988 primers were able to detect the proper amount of CVI988 plasmid present in the mixture when the load of CVI988 plasmid was  $1 \times 10^4$  or higher. When the load of CVI988 plasmid was of  $1 \times 10^3$ , CVI988 primers could still detect the proper amount of CVI988 present in the mixture as long as the load of oncogenic plasmid was not higher than  $1 \times 10^2$ . (B) A fixed amount of oncogenic plasmid was mixed with serial dilutions of the CVI988 plasmid, and mixtures were amplified with

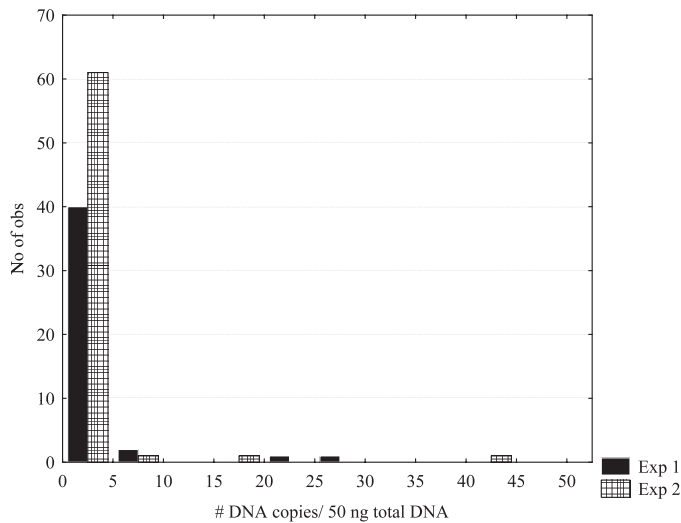


Fig. 5. CVI988 DNA load in FP samples. Feather pulp samples (108) were obtained from two different experiments (Experiments 1 and 2; see Tables 2, 3). Samples tested came from chickens that had been vaccinated with CVI988 either *in ovo* or at 1 day of age with various doses of the vaccine and were not challenged. In addition, samples had been collected at different ages (1, 3, and 8 wk of age). Load of CVI988 DNA was calculated using CVI988 primers, and results are expressed as number of CVI988 DNA copies per 50 ng of total DNA.

DNA in latently infected tissues is not high enough to affect sensitivity/specificity of CVI988 primers. However, load of oncogenic MDV DNA in tumor samples is high enough to negatively affect the sensitivity/specificity of CVI988 primers.

Correlation of results obtained with gB and with oncogenic primers was very high ( $r = 0.99$ ,  $P < 0.05$ ; results not shown). Tissues latently infected with MDV (defined as ratio  $Ct$  GAPDH/ $Ct$  gB  $< 1.7$ ) had a ratio  $Ct$  GAPDH/ $Ct$  oncogenic  $< 1.17$ . On the other hand, tumors induced by MDV (defined as ratio  $Ct$  GAPDH/ $Ct$  gB  $\geq 1.7$ ) had a ratio  $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$ . For the remaining part of the study, we considered  $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$  as the cutoff point for MD tumors when using oncogenic primers.

**Optimization of sampling time when using MAMA primers.** To determine how early in the pathogenesis of the disease oncogenic MDV DNA load could reach levels compatible with tumors (ratio  $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$ ) and therefore affect the sensitivity/specificity of the CVI988 primers, FP samples from three experiments (Experiments 3–5) were used. Chickens from those experiments were vaccinated with HVT or HVT+SB-1 and challenged at 1 day of age by contact either with 648A or Md5. Load of oncogenic MDV was evaluated by using oncogenic primers, and tumor level was set at the ratio  $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$ . Results are presented in Fig. 6. None of the evaluated samples collected at 1 wk of age (150 FP samples) had tumor levels. However, a variable percentage (8%–49%) of samples collected at 3 wk of age and had levels compatible with tumors. Because CVI988 primers can detect oncogenic MDV when the load of oncogenic MDV is compatible with tumors, our results show that for

Table 5. Oncogenic MDV DNA load in tissues latently infected with MDV or in tumors induced by MDV.

Type of infection	Sample	Oncogenic primers		CVI988 primers	
		$Ct$ ratio	No. of copies	$Ct$ ratio	No. of copies
Latency <sup>A</sup>	L1	0.99	9.01	neg <sup>B</sup>	neg
	L2	0.98	6.47	neg	neg
	L3	0.97	3.84	neg	neg
	L4	0.97	3.45	neg	neg
	L5	0.92	2.05	neg	neg
	L6	0.91	1.78	neg	neg
	L8	0.87	0.54	neg	neg
	L9	0.83	0.2	neg	neg
	L10	0.83	0.17	neg	neg
	L11	0.8	0.01	neg	neg
	L12	0.78	0.01	neg	neg
Tumors <sup>C</sup>	T1	1.45	858	0.93	9.16
	T2	1.36	593	1.04	4.01
	T3	1.34	548	0.89	4
	T4	1.3	519	0.89	3.55
	T5	1.24	439	0.99	3.39
	T6	1.21	438	1.07	3.19
	T7	1.19	255	0.9	1.18
	T8	1.18	238	0.92	0.69
	T9	1.18	214	0.89	0.3
	T10	1.17	174	0.86	0.14
	T11	1.17	161	0.9	0.11
	T12	1.17	156	0.81	0.05

<sup>A</sup>Latently infected tissues from chickens vaccinated with serotypes 2 and 3 vaccines and properly protected against a virulent virus infection. Latency was confirmed by real-time PCR using gB primers as described (12,21,22). Latency levels using gB primers correspond to ratio  $Ct$  GAPDH/ $Ct$  gB  $< 1.7$ .

<sup>B</sup>neg = negative.

<sup>C</sup>Tumors samples from chickens vaccinated with serotypes 2 and 3 vaccines and challenged with vv+MDV strain that develop gross tumors. Tumors were confirmed by real-time PCR using gB primers as described (12,21,22). Tumor levels using gB primers correspond to ratio  $Ct$  GAPDH/ $Ct$  gB  $\geq 1.7$ .

monitoring CVI988 using MAMA primers, samples should be collected at 1 wk of age. On the other hand, the use of oncogenic primers could be done at any time because oncogenic primers cannot detect CVI988 at the low levels present within *in vivo* samples.

**Monitoring CVI988 vaccination using MAMA primers in chickens vaccinated with CVI988 and challenged.** Load of CVI988 DNA in FP samples from 1-wk-old chicken flocks was evaluated using nine cases submitted to our laboratory from commercial farms for monitoring CVI988 vaccination (Fig. 7). The percentage of samples positive for CVI988 in those flocks was compared with the percentage of positive samples found in commercial chickens (both meat type and egg type) inoculated experimentally in our laboratory with various CVI988 strains, following the manufacturer's recommendations in three different trials. CVI988 was detected with the CVI988 primers in 50%–80% of the chickens that were vaccinated at the laboratory. Similar levels of CVI988 detection was found in five out of nine submission cases (Case 5 to Case 9). The percentage of chickens in which CVI988 was detected in the other four cases (Case 1 to Case 4) was lower ( $P < 0.05$ ) than the rest of

oncogenic primers. The expected level of oncogenic present in the mixture (dotted line) was compared with the detected level of oncogenic (black solid line). Oncogenic primers were able to detect the proper amount of oncogenic plasmid present in the mixture as long as the load of oncogenic plasmid was  $1 \times 10^2$  or higher. When the load of oncogenic plasmid was of  $1 \times 10^1$ , oncogenic primers could still detect the proper amount of oncogenic plasmid present in the mixture as long as the load of CVI988 plasmid was not higher than  $1 \times 10^6$ .



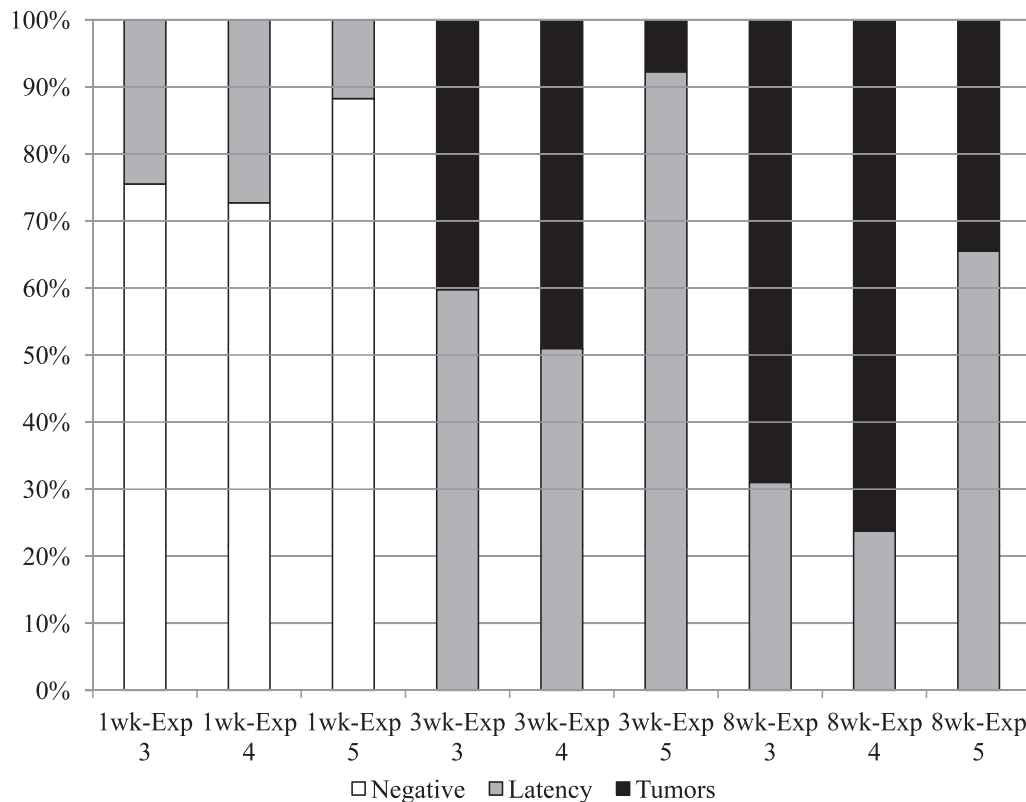


Fig. 6. Load of oncogenic DNA in FP samples. 450 FP samples from three experiments (Experiments 3–5) were used. Chickens on those experiments were vaccinated with HVT or HVT+SB-1 and challenged at 1 day of age by contact either with 648A or Md5 were used. Samples were collected at 1, 3, and 8 wk of age. Load of oncogenic MDV was evaluated by using oncogenic primers, and tumor level was set at ratio *Ct* GAPDH/*Ct* oncogenic  $\geq 1.17$ . Chickens were classified based on the *Ct* ratio into three categories: negative, latently infected, and bearing tumors, as explained in the Materials and Methods section. A chicken was considered negative when the FP sample was amplified for the GAPDH but not for oncogenic pp38 gene; latency when the *Ct* ratio GAPDH/oncogenic was lower than 1.17; and tumor when the *Ct* ratio GAPDH/oncogenic was higher or equal to 1.17.

the groups (17%–36%), suggesting that CVI988 might not be replicating properly in those flocks.

*Using MAMA primers for MD diagnosis in chickens vaccinated with CVI988 and challenged.* To see if MAMA primers could also be used for early diagnosis of MD in FP samples at 3 wk of age, we evaluated the load of oncogenic MDV in FP samples from three experiments (Experiments 6–8) using oncogenic primers. The percentage of chicken with tumor levels (ratio *Ct* GAPDH/*Ct* oncogenic  $\geq 1.17$ ) was evaluated in the different treatment groups. The percentage of tumors in the FP samples collected at 3 wk was correlated with the PI obtained at the end of the experiments (8 wk) for that particular treatment group (Fig. 8). Correlation between the percentage of tumors in the FP collected at 3 wk and the PI was very high ( $r = 0.9$ ,  $P < 0.05$ ), showing that the oncogenic primers can be used for early diagnosis of MD.

In addition, oncogenic primers were used to confirm the diagnosis of MD in 99 samples submitted to the laboratory. All samples were imprints of tumor tissues collected in FTA® cards, and there were a total of eight submissions. Diagnosis of MD was confirmed in six out of the eight submissions using oncogenic primers, gB primers, and histopathology. The other two cases were not diagnosed as MD by any of the three techniques. The agreement between the three techniques was 100%.

## DISCUSSION

A real-time PCR assay that allows differentiation between CVI988 and other serotype 1 MDV has been developed in this

study. We have validated this assay *in vitro* and *in vivo* and demonstrated that can be used for monitoring CVI988 vaccination and for MD diagnosis. Several attempts to develop an assay that permit differentiation between CVI988 and other serotype 1 MDVs have been done in the past (5,31,34). Monoclonal antibodies H19 and T65 were to date the most efficient way to differentiate between them (14). PCRs and real-time PCRs assay also have been described (5,31). However, such assays could be only used under laboratory conditions (5) or in very distinct geographic areas (31).

Monoclonal antibodies H19 and T65 have been used to differentiate CVI988 from other serotype 1 MDVs. Monoclonal antibody H19 recognizes all serotype 1 MDVs except for CVI988. T65 recognizes CVI988 but does not recognize most other serotype 1 MDVs. GA/22 is an exception, and T65 is positive in an immunofluorescence assay with GA/22-infected cells (13). The SNP at pp38 nucleotide base 326 (Fig. 1C) was reported to be associated with the T65 epitope. Most MDVs have Ade and Glu at this location. CVI988, GA/22 and 571 all have a Gua at position 326 that results in Gly. We recently received six MDV field isolates (701, 703, 709A, 710, 718, and 723) that reacted with T65 (Table 4). Upon DNA sequencing the isolates, we found a Gua at position 326 (data not shown). Thus, immunofluorescence with monoclonal antibody T65 is not a reliable assay to uniquely detect CVI988. The epitope recognized by H19 appears to be associated with the SNP located at nucleotide base 320 in the pp38 gene. CVI988 has a Gua at this position, resulting in Arg, while all other sequenced pp38 genes of MDVs have Ade and Gln.

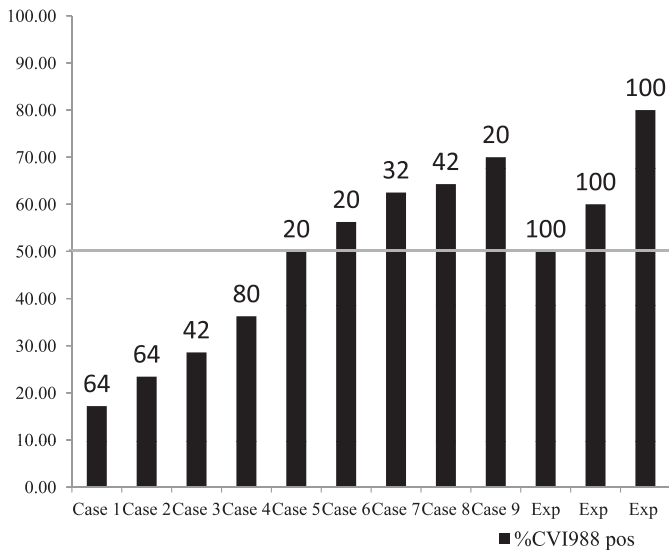


Fig. 7. CVI988 replication in FP samples at 1 wk of age in experimental (Exp) and commercial flocks (Case 1 to Case 9). The bar shows the percentage of samples in which CVI988 was detected using CVI988 primers. The number above the bar indicates the number of samples per submission or experimental group. The grey line was set at the lowest percentage of positive chickens found in experimental groups (50%).

The MAMA PCR targets the SNP at position 320 and is able to detect and differentiate CVI988 from all other tested serotype 1 MDVs. To be reliable, the PCR protocol should be followed exactly. In particular, the primers should be purified with HPLC. We also recommend using JumpStart Taq and to use a minimal amount of template DNA.

We have optimized the use of MAMA primers using *in vivo* samples. Our results demonstrated that there are limitations in the use of these primers due to specificity/sensitivity constraints derived from the difference of just one nucleotide. However, our results show that, if judiciously used, MAMA primers can be very useful for monitoring CVI988 vaccination in the field and for MD diagnosis in flocks vaccinated with CVI988. To the knowledge of the authors, this is the first report of a real-time PCR assay that allows differentiation between CVI988 and other serotype 1 MDV and can be used under field conditions.

Monitoring replication of MD vaccines in the chickens is important to ensure that the vaccination process is done properly. Administration and handling MD vaccines is complicated because MD vaccines are cell associated and are very labile. A variety of factors, such as temperature used for thawing the vaccine, use of antibiotics in the vaccine mixture, diluent used, and interval of time between thawing and vaccination, can affect cell viability and therefore vaccine titers (20). In addition, it is not uncommon that doses lower than recommended by the manufacturer are used to decrease costs (20,26). Measuring replication of the vaccine in the chickens within the first weeks of age is an indirect way to confirm that vaccination was done properly. Traditionally, monitoring replication of MD vaccines has been done by plaque assay, but this technique is time consuming and provides variable results because it depends on the viability of the vaccine virus during processing (9,12). For MD vaccines of serotypes 2 and 3, several real-time PCR methods have been developed and are applied successfully (21,24). In this study, we have developed a real-time PCR assay that allows monitoring of CVI988 replication *in vivo*. We have optimized this assay to be conducted in FP samples collected at

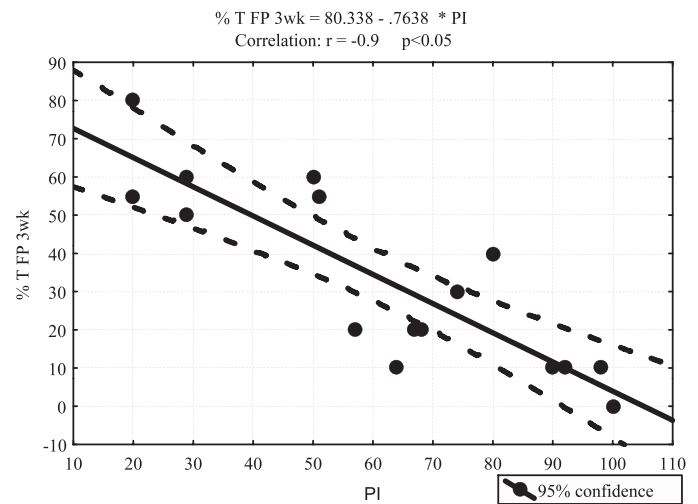


Fig. 8. Early diagnosis of MD using oncogenic primers. Load of oncogenic MDV in FP samples from three experiments (Experiments 6–8) using oncogenic primers was evaluated. The percentage of tumors ( $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$ ) in FP samples collected at 3 wk was correlated with the PI obtained at the end of the experiments (8 wk) for that particular treatment group. Pearson correlation test was used and Pearson correlation was  $r = 0.9$  ( $P < 0.05$ ).

1 wk of age. Age is important for two reasons. First, it can be ensured that no animal at 1 wk of age will have a load of oncogenic DNA compatible with tumors that might affect the specificity of the assay. This is true even in extreme challenge conditions such as chickens challenged at 1 day of age by contact with a vv+MDV strain 648A (as we used to validate this assay). Second, it has been demonstrated that regardless of the vaccine dose used, the vaccine tends to replicate and by 3 wk reach a plateau (4,20). Therefore, to be able to detect problems in the initial replication of the vaccine, samples should be collected in the first week of age. In this study, we have demonstrated that using this assay differences can be found among vaccinated flocks. Under optimum conditions of vaccination (experimental), we found that CVI988 DNA could be detected in the FP of 50%–80% of the animals at 1 wk of age but ranged between 17%–70% in commercial flocks. Several factors might be responsible for such variability, such as vaccine dose, origin of the vaccine, age at vaccination, and chicken strain (1,4,20,42). MAMA primers will allow us to further understand the early pathogenesis of CVI988, to monitor CVI988 replication under different vaccine protocols, and to establish a threshold level of chickens that should be positive for CVI988 at 1 wk of age to achieve optimum vaccination.

In previous work, we have demonstrated that load of challenge MDV DNA in blood can be used as an early criterion of diagnosis of MD (21). Chickens that are poorly protected against MDV had a higher load of MDV DNA in blood or in FP samples than those that are properly protected as early as 3 wk after infection (12,20,21). Our previous studies were conducted in chickens that had been vaccinated with serotypes 2 and 3 vaccines; therefore, the only serotype 1 MDV used was the challenged virus. The real-time PCR technique used for that study is based on amplification of gB that cannot differentiate between CVI988 and other serotype 1 MDV. In the present study, we have demonstrated that MAMA primers (oncogenic primers) can be used in CVI988 vaccinated flocks for early diagnosis of MD. Correlation between the percentage of chickens that had a high load of MDV challenge virus (tumor levels or ratio  $Ct$  GAPDH/ $Ct$  oncogenic  $\geq 1.17$ ) in the FP at 3 wk of age

and the PI observed at 8 wk was very high ( $r = 0.9$ ,  $P < 0.05$ ). This assay could be very useful to reduce the time for vaccine trials (from 8 to 3 wk) as well as to have an indirect evaluation of protection in commercial flocks as early as 3 wk.

Differential diagnosis of poultry tumor diseases can be very complex (22,41). Histopathology is commonly used but sometimes might not be able to provide a conclusive diagnosis. In previous work, we have demonstrated that load of MDV DNA in the tumors is a valid diagnostic criterion for MD (22). In the previous study, we used primers that amplified gB and could not differentiate between CVI988 and the oncogenic virus. This study demonstrated that MAMA primers (oncogenic primers) can be used for confirming MD diagnosis in imprints of tumors collected in FTA cards.

Hence, the results of this work demonstrated that MAMA primers can differentiate between CVI988 and other serotype 1 MDV, and they can be used in biologic samples for monitoring CVI988 replication and for diagnosis of MD in flocks vaccinated with CVI988. The use of MAMA primers is especially important for monitoring CVI988 vaccination. Infection with oncogenic MDV can occur very early in the life of the chickens, and using primers that cannot discriminate between CVI988 and oncogenic viruses will detect both CVI988 and oncogenic virus. Because the use of MAMA primers requires very stringent conditions and numerous controls, standardization of this assay might be complex for nonspecialized laboratories.

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